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COMPOSITION OF THE NITROGENOUS COMPONENTS OF THE BUSH BEAN SEED (*PHASEOLUS VULGARIS*) INCLUDING ISOLATION OF δ-ACETYLORNITHINE

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Abstract—The components of the non-protein nitrogen (NPN) fraction of the mature bush bean seed (*Phaseolus vulgaris*) were quantitatively studied using ion-exchange chromatography. A sizeable part of the NPN consists of non-protein amino acids and γ-glutamyl peptides. Eight such compounds, not widely described in plants, comprise 43% of the Kjeldahl N of this fraction. δ-Acetylornithine was isolated from the NPN and characterized. The amino acid composition of the seed protein shows little relationship to that found in the NPN fraction.

INTRODUCTION

SINCE the advent of chromatographic methods, a number of newly described nitrogenous compounds have been isolated and identified from the non-protein nitrogen (NPN) fraction of the seed of varieties of *Phaseolus vulgaris*. The purpose of this study was to make a complete quantitative investigation of the constituents comprising the NPN and protein-N fractions of the bush bean seed; moreover, it was contemplated that hitherto unidentified nitrogen compounds might be revealed during this investigation.

RESULTS AND DISCUSSION

The ninhydrin-positive constituents of the non-protein nitrogen (NPN) fraction were separated and identified by a two-column ion-exchange chromatographic system employing two separate temperature programs with the acid-natural column.[1] Hydrolysis or oxidative alteration of the molecule or of the masking components permitted estimation of some of the obscured peaks.

Results of the analysis of the NPN fraction of the bush bean seed are shown in Table 1. The values are expressed on the basis of the natural air-dry weight of the mature seed. The moisture content was determined to be 7·1% after drying in a forced-air oven at 80°.

The increase in glutamic acid from 3·73 to 28·5 μM/g following strong acid hydrolysis cannot be fully accounted for in terms of the degradation of the enumerated γ-glutamyl peptides and glutamine (Table 1). Only 19·2 μM/g or 67% of the increase in glutamic acid can be related to identified constituents. On the basis of increases of other amino acids after acid hydrolysis of the NPN fraction, it may be implied that other γ-glutamyl dipeptides or larger glutamic acid-containing peptides are present in the NPN fraction.

Following hydrolysis, only 55% of the leucine and 53% of the methionine can be accounted for as being derived from their glutamyl dipeptides. Of those amino acids whose glutamyl dipeptides have not been identified in the NPN of *Phaseolus vulgaris*, β-alanine, lysine, α-amino-n-butyric acid, cystine and glycine demonstrate the most impressive increases

[1] R. M. ZACHARIUS and E. A. TALLEY, *Anal. Chem.* **34**, 1551 (1962).

TABLE 1. NITROGEN COMPOUNDS OF THE NON-PROTEIN NITROGEN FRACTION OF THE BEAN SEED (*P. vulgaris* VAR. TENDERGREEN)

Compound	μg/g Seed*	μM/g Seed*	After 6 N HCl hydrolysis (μM/g seed*)
δ-Acetylornithine	92·0	0·528	0·0
Alanine	97·3	1·09	1·67
β-Alanine	14·5	0·162	1·66
α-Amino-n-butyric acid	9·69	0·094	0·500
γ-Amino-n-butyric acid	2·43	0·024	0·056
Ammonia	32·4	1·91	—
Arginine	818·0	4·70	4·70
Asparagine	620·0	4·69	0·0
Aspartic acid	796·0	5·98	11·3
Choline	1300·0	10·73	—
Cystine	14·5	0·060	0·563
Ethanolamine	12·6	0·207	0·432
Galactosamine†	13·0	0·073	0·0
Glucosamine	12·8	0·071	0·0
Glutamine	155·0	1·06	0·0
Glutamic acid	549·0	3·73	28·5
γ-Glutamylleucine	362·0	1·39	0·0
γ-Glutamylmethionine	141·0	0·506	0·0
γ-Glutamyl-*S*-methylcysteine	3026·0	11·5	0·0
γ-Glutamyl-*S*-methylcysteine sulfoxide	284·0	1·01	0·0
Glycine	32·3	0·430	1·78
Histidine	164·0	1·05	1·24
Homoserine	17·0	0·143	0·143
Hydroxylysine†	6·62	0·041	0·060‡
Leucine	29·0	0·221	2·76
Isoleucine	38·5	0·293	0·536
Allo-Isoleucine	7·05	0·054	0·086
Lysine	16·21	0·111	0·427
Methionine	11·2	0·075	1·04
Methionine sulfoxide	23·4	0·142	0·339‡
S-Methylcysteine	169·0	1·25	15·4
S-Methylcysteine sulfoxide	49·4	0·326	0·326‡
1-Methylhistidine†	2·68	0·158	0·158
3-Methylhistidine†	tr	tr	tr
Ornithine	2·60	0·020	0·536
Phenylalanine	51·2	0·310	0·605
Pipecolic acid	6788·0	52·6	52·6
Proline	76·0	0·660	1·07
Serine	58·8	0·560	1·11‡
Threonine	43·7	0·366	0·596
Trigonelline	100·0	0·729	0·729
Tryptophan	91·5	0·448	0·256‡
Tyrosine	21·4	0·118	0·175
Valine	73·3	0·625	0·986

* Values are on the basis of the natural air-dry wt. of the seed.
† Tentatively identified by elution volume, 440/570 nm absorption ratio and stability to hydrolysis.
‡ After 2·9 N HCl hydrolysis with which a higher value was obtained.
tr = trace.

following acid hydrolysis of the fraction. Lesser increases, though still exceeding 50%, were obtained with serine, alanine, tyrosine, threonine, valine, proline, isoleucine, ethanolamine, phenylalanine and γ-aminobutyric acid.

The major portion of the increase of cystine could be traced to a component lying within the γ-glutamyl-*S*-methylcysteine peak which was not glutathione. The cystine may be attributed to the presence of γ-glutamylcysteine but the disparately large quantity of γ-glutamyl-*S*-methylcysteine did not permit a precise assignment of glutamic acid resulting from hydrolysis of the components of this region of the chromatogram. Moreover, one cannot ascribe the cystine increase to homoglutathione[2] because the tenfold increase in β-alanine did not occur in the same chromatographic fraction. Glutathione and homoglutathione have been described in seedlings or other actively metabolizing tissue and not in dormant seeds. Presence of storage forms of β-alanine in mature seed may be pertinent to the finding of β-alanine aminotransferase in the cotyledons of wax bean seedlings.[3]

Increases in the aromatic amino acids probably arose from the hydrolysis of small peptides in the NPN but not their γ-glutamyl dipeptides. The rise in α-aminobutyric acid and some of the glycine may be artifacts of acid hydrolysis.[4]

The asymmetrical "serine" peak was reduced in size and became symmetrical after acid hydrolysis. When the original peak was removed from the effluent stream and hydrolyzed, it was found that no more than 58% of the components represented serine. Other hydrolytic products were glutamic acid, glycine, and alanine in a ratio very close to 1:2:1 suggesting some small peptide(s). A prominent unidentified compound, eluting before glycine, was stable to strong acid hydrolysis and to hydrogen peroxide.

Trier[5] first recognized ethanolamine as a constituent of bean seed phosphatides. In this study, the compound was found to represent nearly one-half of the total ethanolamine content in the seed.

Arginine is the major component of the basic fraction of the NPN. A number of small unidentified peaks were observed, three of which were conditionally identified as hydroxylysine, 1-methylhistidine and 3-methylhistidine. The largest basic unidentified peak, immediately preceding arginine, was stable to vigorous acid hydrolysis.

Acid hydrolysis of the NPN produced a decided increase in the ornithine, suggesting that it may be derived from a related compound or peptide. δ-Acetylornithine (N^5-acetylornithine) has been isolated from plants[6–9] and evidence presented that α-acetylornithine lies in the pathway of ornithine biosynthesis in *Escherichia coli*.[10] In an earlier study of the chromatographic behavior of amino compounds according to the procedure employed herein, it was found that δ-acetylornithine had an elution volume identical with asparagine and glutamine.[1] Therefore, the asparagine–glutamine region of the bean NPN fraction was removed before reaction with ninhydrin. On acid hydrolysis followed by column chromatography, ornithine and ammonia were found to be the only sizeable components present.

A scaled-up version of the analytical column was used to yield a quantity of the asparagine–glutamine peak which was further resolved by chromatography on a paper roll.[11] The isolated unknown produced an amount of ornithine, on acid hydrolysis, which could be

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[3] R. A. STINSON and M. S. SPENCER, *Biochem. Biophys. Res. Commun.* **34**, 120 (1969).
[4] R. MARKHAM and J. D. SMITH, *Nature* **164**, 1052 (1949).
[5] G. TRIER, *Z. Physiol. Chem.* **73**, 383 (1911).
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[7] A. I. VIRTANEN and P. LINKO, *Acta Chem. Scand.* **9**, 531 (1955).
[8] L. FOWDEN, *Nature* **182**, 406 (1958).
[9] D. H. BROWN and L. FOWDEN, *Phytochem.* **5**, 881 (1966).
[10] H. J. VOGEL, in *Amino Acid Metabolism* (edited by W. D. MCELROY and B. GLASS), p. 335, Johns Hopkins Press, Baltimore, Maryland (1955).
[11] L. HAGDAHL and C. E. DANIELSON, *Nature* **174**, 1062 (1954).

quantitatively related to the amount of unknown used. All physical and chemical criteria (see Experimental) conclusively show it to be δ-acetyl-L-ornithine.

Brown and Fowden[9] reported that *P. vulgaris* contained a component resembling δ-acetylornithine but which yielded no detectable ornithine on hydrolysis. As is evident from the analytical findings presented in Table 1, δ-acetylornithine is present in relatively low concentration in the bean seed but its hydrolytic product was ascertained on the rather sensitive amino acid analyzer. No α-acetylornithine or γ-acetyldiaminobutyric acid[9,12] was found.

Kjeldahl and Van Slyke N values obtained on the NPN, protein N and the entire seed are given in Table 2. A summing-up of the individual Kjeldahl and Van Slyke N theoretically

TABLE 2. BALANCE SHEET OF N FRACTIONS OF BUSH BEAN SEED (AS mg N/g)

	Summing up of individual compounds	Determined	Accounted (%)
Van Slyke N	0·811	1·02	79·4
Kjeldahl N			
NPN	2·09	2·73	76·6
Protein N	32·6	32·3	100
Entire seed N	—	36·2	—

identified with each of the individual components determined in the NPN is compared with the actual values obtained. In this manner nearly 80% of the Van Slyke N and 77% of the Kjeldahl N could be accounted for in terms of specific components in the NPN of the seed. In a similar summation, all of the Kjeldahl N of the protein fraction can be accounted for.

In a recent paper, Jones and Boulter[13] reported an amino acid analysis of the hydroylzed free and bound nitrogen fractions of the bean seed *P. vulgaris*. Even considering that their analyses were less extensive than in this study, the findings contain major gaps and are not wholly in agreement with the present report. Of the acid-stable components of the NPN, curiously, Jones and Boulter did not report the presence of pipecolic acid or *S*-methylcysteine and apparently failed to identify the smaller quantities of β-alanine, δ-acetylornithine and homoserine. They found no valine, ethanolamine, α-aminobutyric acid or cystine but did provide values for citrulline, an amino acid which we could not confirm in our investigation.

EXPERIMENTAL

Ion-Exchange Chromatography

Quantitative analyses of the amino compounds of the NPN and protein-*N* were carried out on a Phoenix amino acid analyzer, Model K-5000 (no endorsement implied). The resin, length of columns, program temperature, buffers and ninhydrin reagent were nominally the same as those prescribed by Spackman *et al.*[14] The few modifications, including the program timing, determination of elution volumes and 440/570 nm absorption ratios of the ninhydrin products, were described by Zacharius and Talley.[1]

The quaternary nitrogen compounds, trigonelline and choline, were determined by the original method of Christianson *et al.*[15]

[12] I. LISS, *Phytochem.* **1**, 87 (1962).
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[14] D. H. SPACKMAN, W. H. STEIN and S. MOORE, *Anal. Chem.* **30**, 1190 (1958).
[15] D. D. CHRISTIANSON, J. S. WALL, R. J. DIMLER and F. R. SENTI, *Anal. Chem.* **32**, 874 (1960).

Extract Preparation

Mature bush bean seeds (*Phaseolus vulgaris*, var. Tendergreen) were fully extracted with 75% aq. EtOH. All extracts were combined and concentrated on a rotary evaporator at less than 40° under reduced pressure to a volume which represented 1·33 g seed/ml. The fully extracted residue represented the bulk protein.

Hydrolysis

Aliquots of the seed preparation were hydrolyzed in sealed tubes which had been flushed with purified N_2 and then evacuated. Aliquots of the NPN fraction were hydrolyzed in the autoclave at 15 lb with 2·9 N HCl for 10 hr or with 6·0 N HCl for 22 hr. The protein N fraction was hydrolyzed under the latter conditions. The HCl was fully removed on the rotary evaporator under reduced pressure.

Oxidation of S-Containing Compounds

The NPN fraction was treated with sufficient H_2O_2 to provide a 6% H_2O_2 solution. Adding a trace of catalase to the oxidized preparation destroyed the excess H_2O_2 without interfering with the subsequent chromatographic analysis.

Removal of Compound(s) from Effluent Stream of Analytical Column

Once the elution volume was ascertained, the desired component was diverted from the effluent stream before mixing with ninhydrin and entering the reaction coil. The volume in the line between the analyzer's manifold and the photocell was determined and the diversion of the effluent stream via the manifold stopcock was commenced in advance of the peak's expected appearance on the chart. The volume of effluent tapped off depended on the peak spread and degree of resolution from surrounding peaks.

In this manner, those elution volumes from the 150-cm column containing serine (30–50° program), aspartic acid (50°), γ-glutamyl-*S*-methylcysteine (50°), γ-glutamylleucine (50°) and *S*-methylcysteine (50°) were removed and examined.

Isolation of δ-Acetylornithine

Isolation was carried out on a column of Dowex-50 × 8 (<200 mesh) with a capacity 24 times that of the analytical. Three replicate passes were made and the related fractions combined. The desired fractions were desalted on Dowex-2 × 8 (OH^-) (<200 mesh), in the cold, eluting the amino compounds with N acetic acid. The mixture was concentrated under reduced pressure at 38° and further resolved by chromatography on a paper roll[11] with *n*-BuOH–HOAc–H_2O (90:10:25). Collecting 15-ml fractions, δ-acetylornithine appeared in fractions 440–446. Recrystallization from aq. ethanol yielded, after drying, 5·9 mg of pure δ-acetylornithine.

Identification and Criteria of Purity

The isolated compound had a m.p. 255° (N_2, capillary), not depressed when mixed with authentic δ-acetyl-L-ornithine.

On the 150-cm IR-120 column of the amino acid analyzer system at both the 30° and 50° program, a mixture of the isolate and authentic δ-acetyl-L-ornithine yielded a single symmetrical peak with no change in 440/570 nm absorption of the ninhydrin product. Co-chromatography of the isolate with α-acetylornithine produced two separate peaks, the latter partially overlapping methionine.

Descending one-directional chromatography carried out on Whatman No. 1 paper employed the following solvent system: For compound purity and co-chromatography with δ-acetylornithine phenol–H_2O (80:30); *n*-BuOH–HOAc–H_2O (90:10:25) and ethyl acetate–pyridine–H_2O (20:10:10). The latter system also separated 2,3-diaminobutyric acid (R_{ala} 0·79) from ornithine (R_{ala} 0·59).

A 1·0 μM aliquot of the isolated compound hydrolyzed in 6 N HCl for 22 hr in the autoclave under a reduced N_2 atmosphere yielded on the amino acid analyzer 0·95 μM of ornithine. The identity of ornithine was indicated by its 440/570 nm ninhydrin absorption ratio (0·373) whereas that of 2,4-diaminobutyric acid, with a similar elution volume, had a ratio of 0·283. The identity of the hydrolytic product was confirmed with the ethyl acetate–pyridine solvent system combined with the ornithine specific reagent of Curzon and Giltrow.[16]

I.r. spectra of KBr pellets of authentic δ-acetylornithine and the isolated compounds obtained on the Beckman IR-7 were identical and different from that of γ-acetyldiaminobutyric acid.

Acknowledgements—The author is grateful to Mr. Samuel Krulick for the Kjeldahl and Van Slyke nitrogen determinations and to Mr. Jesse Ard for the i.r. absorption spectra. He also thanks Mr. C. J. Morris (U.S. Plant, Soil and Nutrition Laboratory, Ithaca, N.Y.) for the authentic synthetic samples of α- and δ-acetyl-L-ornithine and α- and γ-acetyldiaminobutyric acids.

[16] G. Curzon and J. Giltrow, *Nature* **172**, 356 (1953).